

Phospholipase C- γ Modulates Epithelial Tight Junction Permeability through Hyperphosphorylation of Tight Junction Proteins*

Received for publication, April 2, 2002, and in revised form, June 25, 2002
Published, JBC Papers in Press, July 5, 2002, DOI 10.1074/jbc.M203134200

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Phospholipase C- γ (PLC- γ) is stimulated by epidermal growth factor via activation of the epidermal growth factor receptors. The PLC inhibitor, 3-nitrocoumarin (3-NC), selectively inhibited PLC- γ in Madin-Darby canine kidney cells without affecting the activity of PLC- β . In contrast, inhibitors of PLC- β , hexadecylphosphocholine and U73122, had no effect on the activity of PLC- γ . Inhibition of PLC- γ by 3-NC was associated with an increase in tight junction permeability across Madin-Darby canine kidney cell monolayers, as evidenced by 3-NC-induced decrease in transepithelial electrical resistance and increase in mannitol flux over a concentration range that was inhibitory to PLC- γ . An analog of 3-NC, 7-hydroxy-3-NC (7-OH-3-NC), which was inactive as an inhibitor of PLC- γ , also had no effect on tight junction permeability. Treatment with 3-NC caused punctate disruption in the cortical actin filaments. The PLC- γ inhibitor, 3-NC, but not the inactive analog, 7-OH-3-NC, caused hyperphosphorylation of the tight junction proteins, occludin, ZO-1, and ZO-2. The serine/threonine kinase inhibitor, staurosporine (50–200 nM), significantly attenuated 3-NC-induced hyperphosphorylation of ZO-2. This corresponded with attenuation by staurosporine of 3-NC-induced increase in tight junction permeability, suggesting a relationship between ZO-2 phosphorylation and tight junction permeability.

The concept of the epithelial tight junction as an impermeable barrier has undergone a marked change. Although the rapidly evolving understanding about the architecture and function of the tight junction is far from complete, it is clear that the tight junction is a dynamic and complex multiprotein structure, selectively permeable to certain hydrophilic molecules, ions, and nutrients (1, 2).

The tight junction is a complex structure composed of both transmembrane and cytosolic proteins (3, 4). Many signaling pathways have been implicated in the regulation of tight junction permeability (*e.g.* tyrosine kinases, calcium, protein kinase

C) (5–8). Among the molecular events affected by signal transduction along these pathways, leading to modulation of tight junction permeability, are the phosphorylation status of certain tight junction proteins and organization of actin filaments.

Many of the tight junction proteins (*e.g.* occludin, ZO-1, ZO-2, and ZO-3) are phosphoproteins, and they may regulate tight junction function by alteration of their phosphorylation status (9–12). For example, phosphorylation of occludin is associated with occludin localization and function at the tight junction in Madin-Darby canine kidney (MDCK)¹ cells (13), and increased tyrosine phosphorylation of ZO-1 and ZO-2 is associated with decreased transepithelial electrical resistance (TEER), induced by treatment with a tyrosine phosphatase inhibitor (14). Furthermore, displacement of ZO-1 accompanies the increased tight junction permeability induced by this treatment (6).

In this study, we report that selective inhibition of phospholipase C- γ (PLC- γ), an important class of signaling enzymes, by 3-nitrocoumarin (3-NC) (15) leads to increased tight junction permeability across MDCK cell monolayers. Furthermore, our results show that the mechanisms underlying the increased tight junction permeability following inhibition of the two PLC isozyme families, β and γ , are quite distinct. Thus, inhibition of PLC- γ , unlike inhibition of PLC- β , causes hyperphosphorylation of the tight junction proteins, ZO-1, ZO-2, and occludin.

EXPERIMENTAL PROCEDURES

Reagents—HEPES was obtained from Lineberger Comprehensive Cancer Center (University of North Carolina at Chapel Hill). Hank's balanced salt solution was obtained from Mediatech (Herndon, VA). Cell culture reagents were obtained from Invitrogen. Transwell[™] inserts (12 wells/plate, 3- μ m pore and 1.0-cm² area with polycarbonate or polyester membrane) and plates (12-well) were obtained from Costar (Cambridge, MA). [¹⁴C]mannitol and [³H]myo-inositol were obtained from American Radiolabeled Chemicals (St. Louis, MO). AG1-X8 formate columns were obtained from Bio-Rad. Anti-ZO-1, anti-ZO-2, anti-occludin, rabbit IgG conjugated with horseradish peroxidase, tetramethylbenzidine single solution, and membrane blocking solution were obtained from Zymed Laboratories Inc. (South San Francisco, CA). NuPAGE[™] 4–12% Bis-Tris gels, MOPS running buffer, NuPAGE[™] transfer buffer, and nitrocellulose membranes were obtained from Invitrogen. Phalloidin conjugated with Texas Red[™] was obtained from Molecular Probes (Eugene, OR). Titanium tetrachloride (TiCl₄), salicylaldehyde, ethyl nitroacetate, and *N*-methylmorpholine were obtained from Aldrich. All other compounds and reagents were obtained from Sigma.

¹ The abbreviations used are: MDCK, Madin-Darby canine kidney; PLC, Phospholipase C; 3-NC, 3-nitrocoumarin; 7-OH-3-NC, 7-hydroxy-3-NC; HPC, hexadecylphosphocholine; EGF, epidermal growth factor; TEER, transepithelial electrical resistance; MOPS, 3-(*N*-morpholino)propane sulfonic acid.

* This work was supported by PhRMA Foundation in the form of a Predoctoral Fellowship (to P. W.) and by GlaxoWellcome (unrestricted gift). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Synthesis of 3-NC and 7-OH-3-NC—A procedure adapted from a previously published method was used to synthesize 3-NC and 7-hydroxy-3-NC (7-OH-3-NC) (15). ^1H NMR spectra for 3-NC and 7-OH-3-NC were consistent with those reported (15).

Cell Culture—MDCK epithelial cell line strain II was obtained from the American Type Culture Collection through the Lineberger Comprehensive Cancer Center (University of North Carolina at Chapel Hill). MDCK cells, derived from normal proximal kidney epithelial cells of a male cocker spaniel, which are a model for transport experiments (16), were grown on Transwell™ inserts with polycarbonate membranes for use in studies to determine the effect of absorption enhancers on TEER and mannitol transport. The cells were grown on Transwell™ inserts with transparent polyester membrane for use in confocal studies. Each well was seeded with cells at a density of 100,000 cells/cm². The cells were then grown in cell medium (minimal essential medium supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.2 $\mu\text{g}/\text{ml}$ amphotericin B) and were maintained at 37 °C and 5% CO₂. Confluent cells were grown for 4 days when they differentiated into epithelial cell monolayers as evidenced by the establishment of a stable TEER between 150 and 250 ohm-cm² (EVOM epithelial tissue volt ohmmeter (World Precision Instruments, Sarasota, FL) and an Endohm-12 electrode).

Determination of the Effect of 3-NC on TEER—Cell media were aspirated from both apical and basolateral compartments of Transwell™ inserts and replaced with 0.5 and 1.5 ml, respectively, of transport buffer (Hank's balanced salt solution supplemented with 10 mM HEPES, pH 7.4). MDCK cell monolayers were then incubated at 37 °C for 30 min, and TEER was measured. Experiments were initiated by replacing the media in the apical compartment with transport buffer containing 3-NC or vehicle. MDCK cell monolayers were incubated at 37 °C, and TEER values were measured after 30 min. Data from each experiment were normalized to the response from the vehicle and were reported as the mean \pm S.D. of three experiments ($n = 3$) performed in triplicate. The effect of 3-NC on TEER was evaluated at several concentrations, and the EC₅₀ value, defined as the concentration that caused a 50% decrease in TEER with respect to the untreated control (17, 18), was determined.

Determination of the Effect of 3-NC on Mannitol Transport—Cell media were aspirated from both compartments of Transwell™ inserts and replaced with transport buffer. MDCK cell monolayers were then incubated at 37 °C for 30 min. The integrity of the tight junctions of cell monolayers was monitored with the measurement of TEER prior to the experiment (150–250 ohm-cm²). Transport experiments were initiated by replacing the apical media with 0.5 ml of transport buffer containing 3-NC or vehicle and [^{14}C]mannitol (55 mCi/mmol, 25 μM). Transport rates were monitored by quantifying the amount of [^{14}C]mannitol accumulated (Packard Tri Carb 4000 Series liquid scintillation spectrophotometer) in the basolateral side (1.5 ml) between a 30–60 min interval after initiating the treatment. All transport experiments were conducted under sink conditions (less than 10% of the total amount of [^{14}C]mannitol was present on the basolateral side at any given time). The effect of 3-NC on the transport of [^{14}C]mannitol was normalized to that in the vehicle-treated cells and was reported as the mean \pm S.D. of three experiments ($n = 3$) performed in triplicate. The EC_{10x}, defined as the concentration of 3-NC that causes a 10-fold increase in mannitol flux with respect to the vehicle-treated control (17, 18), was determined from plots of mannitol flux versus 3-NC concentrations.

Determination of PLC Activity and Its Inhibition by Cellular Assay—The activity of PLC- β and - γ in MDCK cells was determined by adaptation of a previously published method (19). MDCK cells were seeded at 400,000 cells/well in a 12-well plate and subsequently cultured for 4 days. The cell monolayers were then labeled with [^3H]myo-inositol (1.6 $\mu\text{Ci}/\text{well}$ in 0.4 ml of inositol-free media) for 24 h at 37 °C. The inhibitor, 3-NC, was added at different concentrations to the media, and the cells were incubated for 30 min at 37 °C. Assays for PLC activity were initiated on labeled cells that were removed from the incubator by immediately supplementing the cells with 100 μl of 250 mM HEPES (pH 7.3), containing 100 mM LiCl with epidermal growth factor (EGF) (final concentration: 20 ng/ml) or ATP (final concentration: 100 μM), which was added to stimulate PLC- γ or - β activity, respectively. The cells were then incubated at 37 °C for 15 min to allow accumulation of [^3H]inositol phosphates. Incubations were terminated by aspiration of the media and the addition of 1 ml of boiling 10 mM EDTA (pH 8.0). The supernatant was applied to AG1-X8 formate columns for chromatographic isolation of [^3H]inositol phosphates (20). The amount of [^3H]inositol phosphates was measured by liquid scintillation counting in a Packard Tri Carb 4000 Series spectrometer. Data from each experiment were normalized to the response observed with 20 ng/ml EGF or 100 μM ATP

for PLC- γ or - β activity, respectively, and were reported as the mean \pm S.D. of three experiments ($n = 3$) performed in triplicate. Inhibition of PLC- β or - γ activity by the respective inhibitors was determined by incubation of the cells with the inhibitors at indicated concentrations for 30 min prior to assaying for the enzyme activity and comparing the enzyme activity so determined with the enzyme activity in untreated control cells. The IC₅₀ (PLC), defined as the concentration of 3-NC that causes a 50% decrease in EGF-stimulated PLC- γ activity (accumulation of [^3H]inositol phosphates), was determined from plots of PLC- γ activity versus 3-NC concentration. The cell viability upon the inhibition treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (21), also known as the MTT assay.

Determination of the ZO-1 and ZO-2 Phosphorylation Status by Autoradiography—MDCK cells were incubated in phosphate-free medium for 60 min at 37 °C. [^{32}P]orthophosphate (1.0 mCi) was added to each flask and incubated for 2 h at 37 °C. 3-NC (PLC- γ inhibition) was added to media at different concentrations, and cells were incubated for 30 min at 37 °C. The media were removed, and 1.5 ml of lysis buffer containing 20 mM Tris (pH 7.5), 2 mM EDTA, 137 mM NaCl, 1% Triton X-100, 10% glycerol with phosphatase and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 $\mu\text{g}/\text{ml}$ leupeptin, 0.2 μM microcystin) were added to each flask. The flasks were then incubated for 1 h at 4 °C. Lysis buffer was transferred into 1.7-ml centrifuge tubes. Agarose-protein A (40 μl) was added to each tube. Tubes were tumbled for 40 min at 4 °C and centrifuged at 15,000 rpm for 1 min. Supernatant was retained and transferred into new 1.7-ml centrifuge tubes, and precipitate was discarded. Anti-ZO-1 or anti-ZO-2 (1 μg) was added to each tube, and the tubes were tumbled for 120 min at 4 °C. Agarose-protein A (40 μl) was added to the tubes with anti-ZO-1 or anti-ZO-2, and the tubes were tumbled for 40 min at 4 °C and then centrifuged at 15,000 rpm for 1 min. Supernatant was discarded, and the immunoprecipitate was washed four times with lysis buffer. SDS gel-loading buffer (40 μl) containing 50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol was added to each tube. Tubes were incubated for 4 min at 75 °C in a dry bath incubator, cooled on ice for 5 min, and centrifuged at 15,000 rpm for 1 min. Samples were subjected to SDS-PAGE. The gels were stained and fixed with colloidal blue in 10% methanol and subsequently dried and exposed to Fuji Medical x-ray Film at -80 °C.

Determination of the Occludin Phosphorylation Status by Western Blotting—MDCK cell monolayers were treated with 3-NC, and occludin was immunoprecipitated with anti-occludin (1 μg) by a procedure identical to that described above. Proteins in the immunoprecipitate were separated on NuPAGE™ 4–12% Bis-Tris gels and subsequently transferred to a nitrocellulose membrane. The membrane was incubated with membrane blocking solution overnight at 4 °C, incubated with anti-occludin (1 $\mu\text{g}/\text{ml}$) for 1 h at room temperature, and then washed with PBS. The membrane was further incubated with goat anti-rabbit IgG, conjugated with horseradish peroxidase for 40 min at room temperature, and then washed with PBS. Tetramethylbenzidine Single solution was then incubated with the membrane until protein bands were visible.

Determination of Actin Organization with Phalloidin—Cell media were aspirated from both apical and basolateral compartments of Transwell™ inserts with transparent polyester membrane and replaced with transport buffer. MDCK cell monolayers were then incubated at 37 °C for 30 min, and TEER of each cell monolayer was measured. Transport buffer containing 3-NC or vehicle was added to the apical side of these cells. The cell monolayers were incubated at 37 °C, and TEER values were measured after 30 min. Transport buffer was replaced with phalloidin conjugated with Texas Red™ (final concentration: 200 nM) in 3.7% formaldehyde (0.2 ml), incubated for 30 min at 4 °C, and subsequently washed with transport buffer. Actin filament organization was viewed with Zeiss LSM-410 inverted laser scanning microscope (Carl Zeiss, Oberkochen, Germany), fitted with a $\times 100$ objective. Several vertical sections (x-z) of the cell monolayers were taken to define the top and bottom of this cell monolayer. *En face* sections (x and y) were then selected from the cortical (mid-cell) sections of the cell monolayers.

Data Analysis—The Student's *t* test for unpaired data was used to determine significant differences ($p < 0.05$) between the mean \pm S.D. from untreated and treated MDCK cell monolayers.

RESULTS

PLC- γ Activity in MDCK Cell—EGF, which has been known to activate PLC- γ via the EGF receptors (22), stimulated PLC- γ activity in MDCK cells, as evidenced by a 3-fold increase in

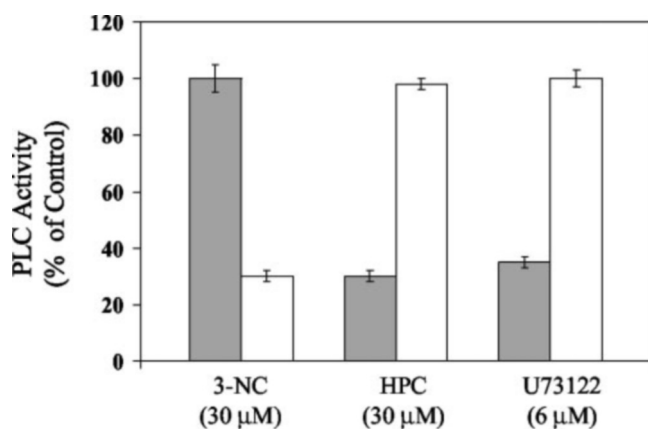


FIG. 1. The selectivity of 3-NC, HPC, and U73122 for inhibition of PLC- β and - γ . MDCK cell monolayers were labeled with [3 H]myo-inositol for 24 h at 37 °C. The effect of the test compounds on PLC- β and - γ activity, stimulated with 100 μ M ATP or 20 ng/ml EGF, respectively, was determined by a 30-min incubation with the cells as described under "Experimental Procedures." Data points represent mean \pm S.D. ($n = 3$). The clear bar represents EGF-stimulated PLC- γ activity, and the shaded bar represents ATP-stimulated PLC- β activity.

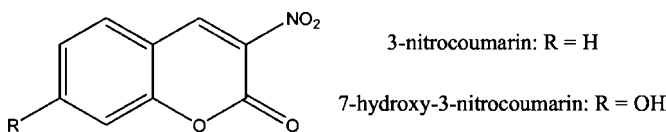


FIG. 2. Structure of the PLC- γ inhibitor, 3-NC, and its inactive analog, 7-OH-3-NC.

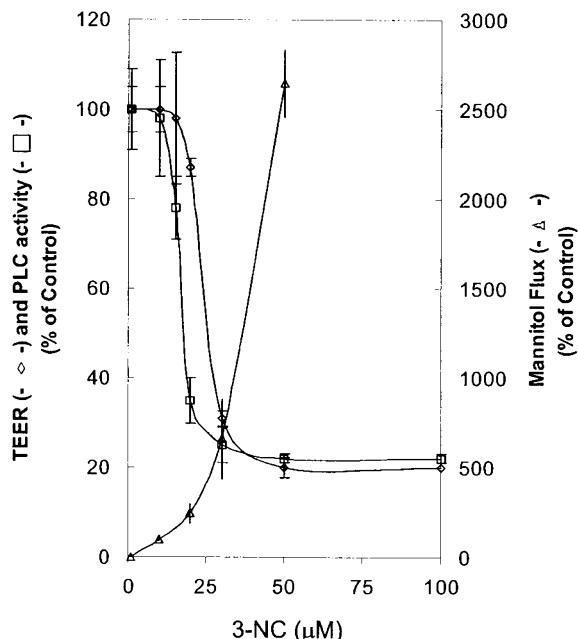


FIG. 3. The effect of 3-NC on TEER, EGF-stimulated PLC- γ activity, and mannitol flux in MDCK cells. TEER was measured after a 30-min incubation with different concentrations of 3-NC at 37 °C. PLC- γ activity was measured as described under "Experimental Procedures." To determine the effect of 3-NC on mannitol flux, [14 C]mannitol and 3-NC were applied apically, and the amount of [14 C]mannitol that accumulated in the basolateral side between 30 and 60 min post-treatment (3-NC or vehicle) was quantified.

[3 H]inositol phosphates over the basal levels in the prelabeled cells. These results established the presence of PLC- γ in MDCK cells. Both U73122 and HPC, known inhibitors of PLC- β ,²

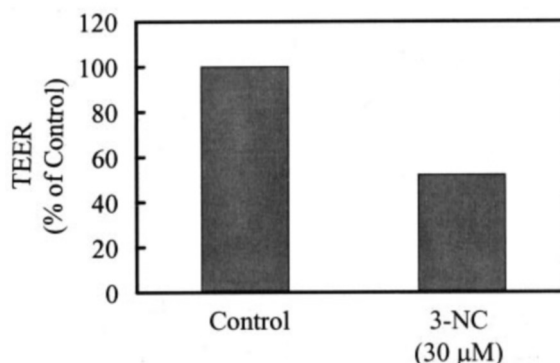
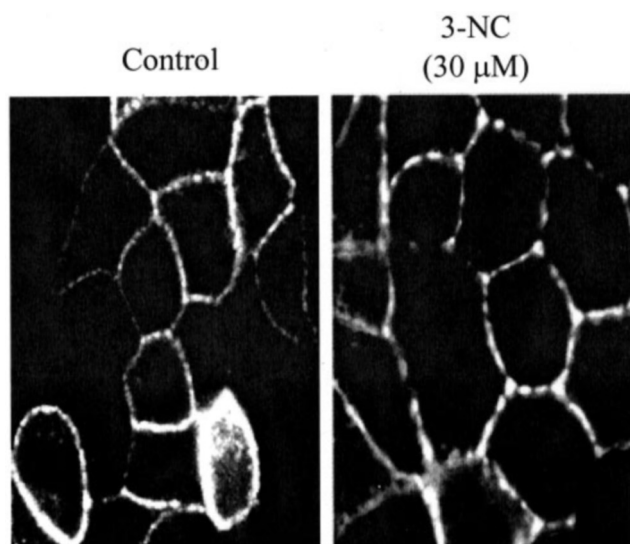


FIG. 4. The effect of 3-NC on actin filament organization in MDCK cell monolayers. The cells were treated apically with 3-NC for 30 min at 37 °C; TEER was measured before and after treatment. Cell monolayers were then incubated with phalloidin-conjugated with Texas Red in 3.7% formaldehyde for 30 min at 4 °C and subsequently washed with transport buffer. Actin filament organization was viewed with Zeiss confocal microscope. Bar = 15 μ m.

inhibited ATP-stimulated PLC- β activity but had no effect on the PLC activity that was stimulated by EGF (Fig. 1). In contrast, 3-NC (see Fig. 2 for structure), a known inhibitor of PLC- γ (15), inhibited EGF-stimulated PLC activity in MDCK cells (30-min treatment) without affecting the ATP-stimulated PLC- β activity (Fig. 1).

Inhibition of PLC- γ and Enhancement of Tight Junction Permeability by 3-NC—The selective PLC- γ inhibitor, 3-NC (15), inhibited the EGF-stimulated PLC- γ activity in MDCK cell monolayers in a concentration-dependent manner (Fig. 3). IC_{50} (PLC), the concentration of 3-NC that inhibited EGF-stimulated PLC- γ activity by 50%, was 18 ± 1 μ M. Interestingly, 3-NC also decreased TEER, a measure of tight junction permeability, across MDCK cell monolayers in a concentration-dependent manner (Fig. 3), and the relationship between decrease in TEER and concentration was very similar to that between decrease in PLC activity and concentration. Furthermore, mannitol flux, which is yet another measure of tight junction permeability, increased progressively when MDCK cell monolayers were treated with increasing concentration of 3-NC on the apical side (Fig. 3). The relationship between 3-NC concentration and increase in mannitol flux corresponded well with the concentration/TEER and concentration/PLC activity relationships (Fig. 3). EC_{50} (the concentration of 3-NC that decreases TEER by 50%) and $EC_{10\times}$ (the concentration of 3-NC that increases mannitol flux by 10-fold) were determined from

² P. D. Ward, H. Ouyang, and D. R. Thakker, unpublished results.

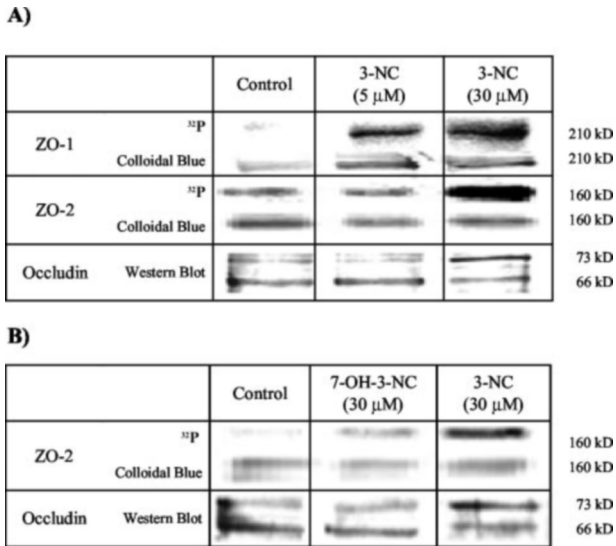


FIG. 5. The effect of 3-NC on the phosphorylation state of ZO-1, ZO-2, and occludin: the effect of 3-NC (A) and the effect of 7-OH-3-NC (B), an inactive structural analog of 3-NC. For determination of ZO-1 and ZO-2 phosphorylation status, 32 P-labeled MDCK cells were treated with the test compound or vehicle (Control) for 30 min. ZO-1 or ZO-2 was immunoprecipitated, subjected to PAGE, stained with colloidal blue, and exposed to Fuji medical x-ray film at -80°C . Protein levels of ZO-1 and ZO-2 for each treatment were similar. For determination of occludin phosphorylation status, MDCK cells were treated with the test compound or vehicle (Control) for 30 min. Occludin was immunoprecipitated and loaded onto a NuPAGE 4–12% Bis-Tris gel, transferred to a nitrocellulose membrane, and incubated with anti-occludin. Subsequently, membranes were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase. Tetramethylbenzidine single solution was added to develop bands.

these relationships, and these values were 24 ± 1 and 34 ± 5 μM , respectively. A close structural analog of 3-NC, 7-OH-3-NC (Fig. 2), which was reported to be inactive as an inhibitor of PLC- γ (15), had no effect on either PLC activity or tight junction permeability across MDCK cell monolayers at a concentration (500 μM) that represented >25 -fold the IC_{50} (PLC) of 3-NC (data not shown). Taken together, these results suggest that inhibition of PLC- γ is associated with an increase in tight junction permeability across MDCK cell monolayers. It is important to note that in the experiments performed to determine the effect of 3-NC on the enzyme activity as well as on TEER and mannitol transport, the cell viability was not compromised as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, also known as the MTT assay (21).

Relationship between PLC- γ Inhibition and Actin Filament Organization.—We have observed that inhibition of PLC- β with U73122 leads to disorganization of actin filaments in the cortical region (mid-cell region) of MDCK cells.² To determine whether inhibition of PLC- γ has a similar effect, MDCK cell monolayers were treated with 3-NC at a concentration that caused a significant increase in tight junction permeability and inhibition of PLC- γ (30 μM). Treatment with 3-NC appeared to have induced punctate disruption in cortical actin as the cortical actin on the cell border appeared more discontinuous than in untreated cell monolayers (Fig. 4). This result suggests that 3-NC might cause changes in the interactions of actin filaments with the proteins in the junctional complexes.

Relationship between Inhibition of PLC- γ and Tight Junction Protein Phosphorylation.—Fig. 5A depicts the phosphorylation status of three tight junction proteins, ZO-1, ZO-2, and occludin, in response to the treatment of MDCK cell monolayers with 3-NC. The phosphorylation status of ZO-1 and ZO-2 was determined by autoradiography of the immunopre-

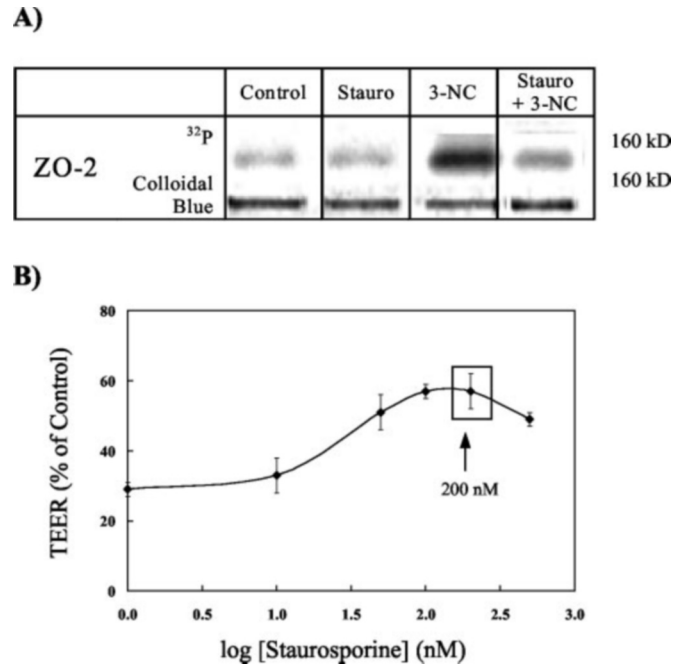


FIG. 6. Co-ordinate attenuation by staurosporine (Stauro) of 3-NC-induced hyperphosphorylation of ZO-2 (A) and increase in tight junction permeability (B). For the phosphorylation experiment, 32 P-labeled MDCK cells were treated with staurosporine (200 nM, maximal effect on TEER (B)) for 1 h at 37°C . Test compound (30 μM) or vehicle was added, and cells were incubated for 30 min at 37°C . Phosphorylation status of ZO-2 was determined as described in Fig. 5. Protein levels of ZO-2 for each treatment were similar. For the TEER experiment, different concentrations of staurosporine were applied apically and incubated for 1 h at 37°C . MDCK cell monolayers were washed with transport buffer, and 3-NC (30 μM) was applied apically. TEER was measured after a 30-min incubation with the test compound at 37°C .

cipitated proteins from 32 P-labeled cells. Whereas the phosphorylation status of occludin was determined by assessing the relative intensity of hyperphosphorylated 73-kDa occludin bands and hypophosphorylated 66-kDa occludin bands (electrophoretic separation and Western blot) by the method of Wong (13).

At a concentration that causes significant inhibition of PLC- γ and increase in tight junction permeability (30 μM), 3-NC caused hyperphosphorylation of ZO-1 and ZO-2 as evidenced by a significant increase (over control) in the intensity of the 32 P bands at 210 and 160 kDa, respectively. Hyperphosphorylation of occludin by this treatment was also apparent from an increase in the intensity of the 73-kDa band and a decrease in the intensity of the 66-kDa band with respect to the vehicle-treated control (Fig. 5A). At a lower concentration (5 μM) that is ineffective in causing PLC inhibition or increased tight junction permeability, 3-NC did not affect phosphorylation status of ZO-2 or occludin; however, phosphorylation of ZO-1 was increased with respect to control. Interestingly, the 3-NC analog, 7-OH-3-NC, which does not inhibit PLC- γ (15) or increase tight junction permeability, was significantly less effective than 3-NC in causing an increase in ZO-2 phosphorylation and had negligible effect on occludin phosphorylation (Fig. 5B). These results suggest that hyperphosphorylation of selected tight junction proteins may be an important step when the tight junction permeability is increased in response to PLC- γ inhibition.

To further investigate the relationship between hyperphosphorylation of these proteins by the PLC- γ inhibitor 3-NC and tight junction permeability across MDCK cell monolayers, the effect of kinase inhibitors on these events was deter-

mined. Tyrosine kinase inhibitors, tyrphostin-25 (24) and genestein (25), had a negligible effect on the 3-NC-induced changes in tight junction protein phosphorylation or permeability (data not shown). On the other hand, the serine/threonine kinase inhibitor, staurosporine (26), significantly attenuated the 3-NC-induced (i) hyperphosphorylation of ZO-2 (Fig. 6A) and (ii) increased tight junction permeability (Fig. 6B). Staurosporine had no effect, however, on 3-NC-induced hyperphosphorylation of occludin (data not shown). These results suggest that 3-NC-induced hyperphosphorylation of ZO-2 in MDCK cells is mediated by a serine/threonine kinase. Unlike 3-NC, the PLC- β inhibitors, U73122 and HPC, did not increase ZO-2 or occludin phosphorylation (data not shown), indicating that PLC- β inhibition does not cause a change in the phosphorylation state of these tight junction proteins.

DISCUSSION

In the present study, we demonstrate that selective inhibition of PLC- γ leads to increased tight junction permeability, presumably via hyperphosphorylation of selected tight junction proteins. In contrast to the selective PLC- β inhibitor, U73122 (27, 28), which caused a marked disruption of actin filaments without causing a change in phosphorylation state of ZO-2 or occludin,² the PLC- γ inhibitor 3-NC (15) caused hyperphosphorylation of the tight junction proteins ZO-1, ZO-2, and occludin. It is important to note that hyperphosphorylation of ZO-2 and occludin is caused by 3-NC at concentrations that are entirely consistent with its potency as an inhibitor of PLC- γ and enhancer of tight junction permeability. The serine-threonine kinase inhibitor staurosporine attenuated 3-NC-induced (i) hyperphosphorylation of ZO-2 and (ii) increase in tight junction permeability across MDCK cell monolayers. These results provide strong support to the hypothesis that hyperphosphorylation of ZO-2 plays a role in increasing the tight junction permeability across epithelial tissues as exemplified by MDCK cell monolayers. Since hyperphosphorylation of ZO-1 occurs at a concentration of 3-NC that does not cause significant inhibition of PLC- γ , the association between PLC- γ inhibition and ZO-1 hyperphosphorylation is somewhat tenuous. However, it is not inconceivable that the extent of hyperphosphorylation caused at suboptimal concentration may not be sufficient to affect sufficient changes in the tight junction structure to cause an observable drop in permeability. Alternatively, 3-NC may cause hyperphosphorylation of a site at a low concentration that is not involved in alteration of the tight junction permeability. A cause-and-effect relationship between phosphorylation of tight junction proteins and increase in tight junction permeability has not been established previously, although some evidence for this does exist. For example, phosphorylation of occludin is associated with reduced tight junction barrier function and assembly in MDCK cell monolayers (13, 29, 30). As with occludin, phosphorylation of ZO-2 has been implicated in reduced efficacy of the tight junction barrier (31–33); for example, the drop in TEER after temperature shift in temperature-sensitive v-src MDCK cells is associated with hyperphosphorylation of ZO-2 (33).

The PLC-dependent pathway is initiated when PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into two intracellular products: diacylglycerol and inositol 1,4,5-triphosphate. Inositol 1,4,5-triphosphate is released into the cytosol and induces Ca^{2+} release from the endoplasmic reticulum (20, 34). Increased levels of diacylglycerol, alone or in conjunction with Ca^{2+} , activate the novel and conventional protein kinase C isozymes (35–37). Thus, PLC appears to play a regulatory role by affecting protein kinase C-mediated alteration of the phos-

phorylation state of the target proteins. The results in the present study, i.e. hyperphosphorylation of the tight junction proteins following inhibition of PLC- γ , are counterintuitive but consistent with the results obtained in other laboratories. For example, activation of protein kinase C by the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate, leads to dephosphorylation of occludin in LLC-PK1 cell monolayers (23). These results suggest that other intervening steps must exist between modulation of the activity of a protein kinase C isozyme and hyperphosphorylation of the tight junction proteins.

In conclusion, the results in this study provide strong evidence for a role of PLC- γ in the regulation of epithelial tight junction permeability. Besides establishing a relationship between the catalytic activity of the enzyme and the tight junction permeability, the results show that the changes in the PLC activity lead to changes in the phosphorylation state of some of the tight junction proteins. The inverse relationship between PLC- γ activity and the phosphorylation state of these proteins suggests the intervention of more than one kinase. The elucidation of events leading to the changes in the phosphorylation state of tight junction proteins following inhibition of PLC- γ will provide more definitive insights into the role of this PLC isozyme in regulation of the structure and function of the tight junction in epithelial tissue.

Acknowledgments—We thank Dr. John Lemasters (Confocal Microscopy Core, University of North Carolina at Chapel Hill) for the use of the Zeiss confocal microscope and Dr. Lee Graves for helpful advice in phosphorylation studies.

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